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Tumorigenic activity of the *BCR–ABL* oncogenes is mediated by *BCL2*

(apoptosis/interleukin 3/leukemia/Philadelphia chromosome/tumor development)

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ABSTRACT ***BCR–ABL* is a chimeric oncogene generated by translocation of sequences from the c-*abl* protein-tyrosine kinase gene on chromosome 9 into the *BCR* gene on chromosome 22. Alternative chimeric proteins, $p210^{BCR-ABL}$ and $p190^{BCR-ABL}$, are produced that are characteristic of chronic myelogenous leukemia and acute lymphoblastic leukemia, respectively. Their role in the etiology of human leukemia remains to be defined. Transformed murine hematopoietic cells can be used as a model of BCR–ABL function since these cells can be made growth factor independent and tumorigenic by the action of the *BCR–ABL* oncogene. We show that the *BCR–ABL* oncogenes prevent apoptotic death in these cells by inducing a *Bcl-2* expression pathway. Furthermore, *BCR–ABL*-expressing cells revert to factor dependence and nontumorigenicity after *Bcl-2* expression is suppressed. These results help to explain the ability of *BCR–ABL* oncogenes to synergize with c-*myc* in cell transformation.**

Hematopoietic growth factors are required to support the proliferation, survival, and differentiation of progenitor cells (1–3). Leukemic conversion of these cells to an autonomous growth state implies that specific genes must be activated to uncouple cellular proliferation/differentiation control and to generate intracellular signals that can substitute for growth factor requirements. A well-characterized example in the hematopoietic system involves the rearrangements of the *BCR* and *ABL* genes in Philadelphia chromosome-positive (Ph^{1+}) chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (4–13). Depending on the precise breakpoint within the *BCR* gene, fusion proteins of 210 kDa (p210) or 190 kDa (p190) are produced (4, 6, 7, 9, 14–16). p210 and p190 *BCR–ABL* oncogenes contain identical *ABL*-derived sequences, respectively, but differ in the number of *BCR*-encoded amino acid residues. The tyrosine kinase activity of the BCR–ABL proteins strongly correlates with their transforming potential in tissue culture and is higher for the 190-kDa form, which is characteristically associated with acute leukemias (12, 14, 17).

Analysis of the mechanism of oncogene action can be studied in cell lines—often in NIH 3T3 fibroblasts. The latter are not transformed by either of the two *BCR–ABL* oncogenes or by v-*abl* itself (18). However, it has been shown that $BCR–ABL^{p120}$ and v-*abl* convert the factor-dependent Ba/F3 cell line into factor independence and make it tumorigenic (19), but the mechanisms whereby this happens are unknown. Understanding these mechanisms requires identification of the downstream involvement of proteins that affect transcriptional control or cell survival. *MYC* is a candidate gene whose transcription is activated by some tyrosine kinase oncogenes as well as by growth factor stimulation (20, 21). Recent data have shown that *MYC* is essential for *BCR–ABL* transformation (22). However, an increase in MYC protein cannot be solely responsible for the transforming effect of *BCR–ABL* oncogenes, because *MYC* overexpression does not substitute for the *BCR–ABL* oncogene transformation phenotype. Therefore, other components in addition to *MYC* must be required to reconstitute the *BCR–ABL* oncogene transformation signal.

In the present study, we have examined the role of *BCR–ABL* oncogenes in the context of hematopoietic cell transformation using as a model system the Ba/F3 cell line (23). We investigated the possibility that *BCR–ABL* expression may function in Ba/F3 cells by inhibiting apoptosis and consequently prolonging cell survival. We show that the *BCR–ABL* oncogenes prevent apoptotic death in these cells by inducing a *Bcl-2* expression pathway. We further show that Ba/F3 cells expressing *BCR–ABL* revert to factor dependence and nontumorigenicity after *Bcl-2* expression is suppressed. These findings implicate the activation of Bcl-2 function as an important component in BCR–ABL-mediated transformation and can also explain the ability of *BCR–ABL* oncogenes to synergize with *MYC* in cell transformation.

MATERIALS AND METHODS

Cell Culture. Cell lines used include Ba/F3 (23), K562 (24), and DoHH2 (25). Ba/F3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3B-conditioned medium as a source of interleukin 3 (IL-3). DoHH2 and K562 cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and 5×10^{-5} M 2-mercaptoethanol.

Plasmid Construction. The pAbc12 plasmid contains the mouse *Bcl-2* cDNA in antisense orientation and the PGK-Hyg cassette into the pEF-BOS expression vector (26). The final product was confirmed by DNA sequence analysis.

Cell Transfection. Ba/F3 cells were transfected by electroporation with 25 μg of KW3 expression plasmid and 25 μg of E1A2 plasmid along with 2 μg of MC1-neo expression vector. Cell lines were analyzed by Northern blotting for *BCR–ABL* expression and demonstrated resistance to IL-3 withdrawal. *BCR–ABL*-expressing Ba/F3 cells were transfected by electroporation (960 μF; 220 V) with 25 μg of the pAbc12 vector. Hygromycin-resistant cells were selected by using 600 $\mu g \cdot ml^{-1}$ of hygromycin B (Calbiochem) for 14 days. Clones were screened for high-level *Bcl-2* expression by Northern blotting. The antisense *Bcl-2* oligonucleotide used as a probe comprises the first 34 bases of the coding sequence of mouse *Bcl-2* cDNA.

RNA Analysis. Total cytoplasmic RNA (10 μg) was glyoxylated and fractioned in 1.4% agarose gels in 10 mM Na_2HPO_4 buffer (pH 7.0). After electrophoresis, the gel was blotted onto

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Abbreviations: CML, chronic myelogenous leukemia; FL, follicular lymphoma; IL-3, interleukin 3.

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Hybond-N (Amersham), UV-cross-linked, and hybridized to ^{32}P-labeled probes.

DNA Analysis. Low molecular weight DNA was isolated as follows. Cells were collected into 1.5 ml of culture medium and microcentrifuged for 5 min at 13,200 rpm (3000 × *g*), and the pellet was suspended in 300 μl of proteinase K buffer. After overnight incubation at 55°C, DNA was ethanol-precipitated, suspended in 200 μl of TE buffer containing 50 μg·ml^{-1} of RNase A, and incubated at 37°C for 2 h. DNA was extracted with phenol and chloroform and precipitated with ethanol. Aliquots of DNA (2 μg) were electrophoresed on 2% agarose gels, which were stained with ethidium bromide and photographed.

Tumorigenicity Assay. To test the tumorigenicity of the various cell lines, 4- to 6-week-old athymic (nude) male mice were injected subcutaneously on both flanks with 10^6 cells resuspended in 200 μl of phosphate-buffered saline (PBS). The animals were examined for tumor formation for up to 2 months.

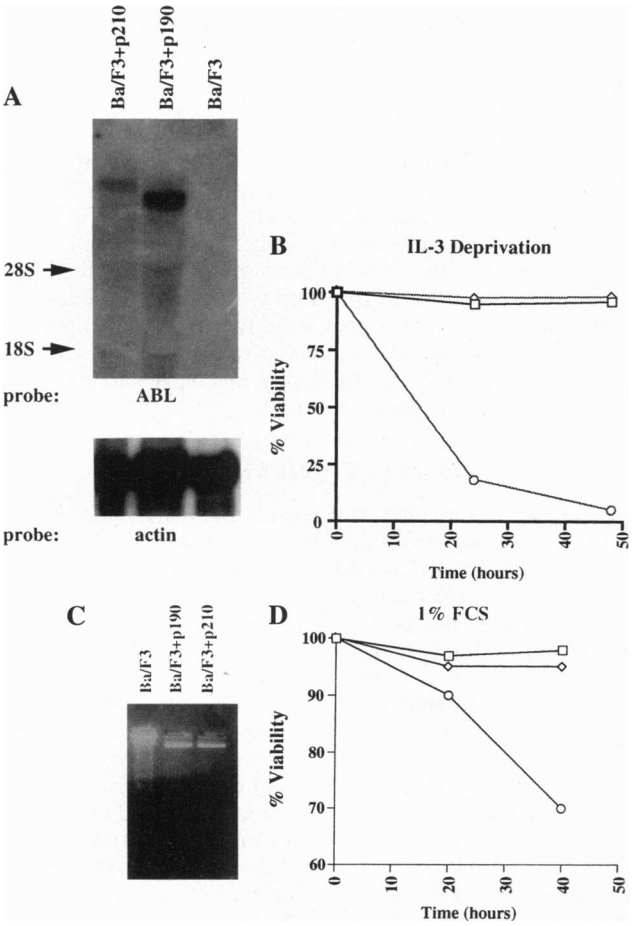

FIG. 1. Inhibition of apoptosis in *BCR–ABL*-expressing Ba/F3 cells. (*A*) Northern filter hybridization analysis of transfected Ba/F3 cell lines. Lanes: 1, Ba/F3 cells transfected with plasmid KW3 expressing BCR–ABL^{p210}; 2, Ba/F3 cells transfected with plasmid E1A2 expressing BCR–ABL^{p190}; 3, untransfected Ba/F3 cell line. Cellular RNA was hybridized to an *ABL* probe. Autoradiography was for 24 h at −70°C. (*B*) Viability of transfected Ba/F3 cells (○) during IL-3 withdrawal. The Ba/F3 cell line is dependent on IL-3 for growth (23) but becomes IL-3 independent when stably transformed by p210 (◇) or p190 (□) cDNAs. (*C*) Cell death is accompanied by nucleosome laddering after IL-3 deprivation. Low molecular weight DNA was isolated 2 days after IL-3 deprivation. DNA was resolved by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. (*D*) Viability of *BCR–ABL*-expressing Ba/F3 cells in low serum medium. Duplicate cultures of cells were cultured in medium containing 1% FCS, and living cells were counted at daily intervals. All values are normalized to the relative viability of the initial number of cells. Percentage viability was determined by trypan blue exclusion. This experiment is one of three that gave similar results.

RESULTS

***BCR–ABL* Oncogenes Inhibit Apoptosis of Ba/F3 Cell Line After IL-3 Withdrawal.** Expression plasmids encoding BCR–ABL^{p190} and BCR–ABL^{p210} proteins were introduced into the Ba/F3 cell line. Stable lines were established and *BCR–ABL* expression was analyzed by Northern hybridization analysis (Fig. 1*A*). *BCR–ABL* transforms the IL-3-dependent Ba/F3 cell line to factor independence and tumorigenicity (19) (Fig. 1*B*; Table 1). The effects of *BCR–ABL* expression on cell growth were evaluated by analyzing internucleosomal DNA cleavage leading to the formation of DNA ladders in agarose gels, which is a hallmark of apoptosis. A DNA cleavage ladder was observed in the Ba/F3 cells undergoing apoptosis in response to IL-3 withdrawal but not in BCR–ABL-expressing Ba/F3 cell lines (Fig. 1*C*). Moreover, transfection of the human *BCR–ABL* oncogenes into the Ba/F3 cell line allowed these cells to survive in the absence of IL-3 not only under optimal (10% FCS) culture conditions but in serum-deprived conditions as well (Fig. 1*D*). These experiments imply that *BCR–ABL* oncogenes, like other transforming genes, protect against programmed cell death rather than, for example, inducing the autocrine expression of a growth factor (19). These results, together with recent data (27, 28), can explain the massive accumulation of cells in the Ph^{1+} human leukemias, where the leukemic progenitors display normal rates of cell proliferation (27–29).

Expression of *Bcl-2* in *BCR–ABL*-Expressing Ba/F3 Cells. The clinical syndromes produced by BCR-ABL^{p210} (CML) and by BCL-2 (follicular lymphoma; FL) are similar, in that both CML and FL exhibit an indolent phase that leads to the development of aggressive malignant phenotypes. Moreover, hematopoietic cell lines transfected with BCL-2 show a relative resistance to the apoptotic death that normally follows growth factor withdrawal (30). Therefore, *BCR–ABL* oncogenes may suppress apoptosis in a manner similar to that seen for the *BCL2* gene in FL (31). We thus examined the possibility that the effect of *BCR–ABL* oncogenes in cell survival could be *BCL2*-mediated. Studies at the mRNA level were performed and results of Northern blot hybridizations are shown in Fig. 2. The human *BCL2* probe detected a major mRNA transcript in both the p190 and p210 *BCR–ABL*-expressing Ba/F3 cell lines similar to the size of that found in the DoHH2 cell line, which contains the typical t(14;18) translocation associated with FL (25). The presence of the mouse Bcl-2 protein was demonstrated by Western blot analysis (data not shown). This also compares to the Ph^{1+}-K562 cell line (24), which expresses the normal-sized *BCL2* transcript of 7.5 kb (Fig. 2*A*) and has the BCL2 protein (32).

***BCR–ABL* Oncogenes Protect Cells from Oxidative Damage.** Apoptosis can be induced in cell cultures by various treatments and if the effect of *BCR–ABL* oncogenes in apoptosis is *BCL2* mediated, protection of cells by BCR–ABL from oxidative damage will be similar to a characteristic BCL2 effect discovered recently (33). Stable transfectants of Ba/F3 cells bearing BCR–ABL were exposed to exogenous hydrogen peroxidase, which kills cells in a dose-dependent manner (Fig. 3). *BCR–ABL*-expressing Ba/F3 cells were completely protected from the lethal effects of 0.25–0.5 mM hydrogen peroxidase (Fig. 3 *A* and *B*). A dose of 1.0 mM resulted in a more rapid cell killing and showed no survival advantage with BCR–ABL (Fig. 3*C*).

Suppression of *Bcl-2* Expression Blocks Transformation by *BCR–ABL* Oncogenes. The above results provide evidence for the idea that *BCR–ABL* expression protects cells from apoptosis by inducing a *Bcl-2* pathway. In that case, some modi-

Table 1. Growth properties of *BCR–ABL*-expressing Ba/F3 cells

Cells	Growth in selective medium		Tumorigenicity in nude mice	
	+IL-3	−IL-3	Tumors/no. of injections	Latency, weeks
Ba/F3	+	−	0/8	
Ba/F3+p210	+	+	8/8	1–2
Ba/F3^{p210}+pAbcl2	+	−	0/8	
Ba/F3+p190	+	+	8/8	1–2
Ba/F3^{p190}+pAbcl2	+	−	0/8	

fication of the growth and tumorigenicity of these *BCR–ABL*-expressing Ba/F3 cells would be expected to occur on altering the level of *Bcl-2* expression. *BCR–ABL*-expressing Ba/F3 cells were transfected with a vector expressing antisense mouse *Bcl-2* cDNA (pAbcl2) and clones were established (Fig. 4*A*). RNA was extracted and the levels of *Bcl-2* mRNA were compared to those in untransfected control clones (Fig. 4*B*). Antisense *Bcl-2* was detected in cells transfected with pAbcl2 but not in untransfected cells and the level of *BCR–ABL* expression was unaffected in the *BCR–ABL*-expressing Ba/F3 cells transfected with pAbcl2 (data not shown). On the other hand, no *Bcl-2* mRNA was detected in the cells transfected with antisense *Bcl-2* in contrast to its presence in Ba/F3^{p190} and Ba/F3^{p210} cells (Fig. 4*B*). In parallel experiments, no Bcl-2 protein was observed in the cells transfected with the antisense *Bcl-2* cDNA (data not shown). The consequences of the suppression of *Bcl-2* expression were evaluated by examining the viability of these cells after IL-3 withdrawal. While the viability of Ba/F3 cells expressing *BCR–ABL* oncogenes is unaffected by the absence of IL-3, those transfected with antisense *Bcl-2* become IL-3 dependent as are Ba/F3 cells (Fig. 5).

The tumorigenicity of the various cell lines was tested by injection into nude mice (Table 1). The initial Ba/F3 cell line is nontumorigenic in this assay, whereas Ba/F3 cells expressing *BCR–ABL* oncogenes grow as tumors in nude mice (19). Likewise, we found that tumor formation was observed as early as 10 days postinjection for *BCR–ABL*-expressing cell lines. In contrast to these controls, Ba/F3 cells expressing both *BCR–ABL* and antisense *Bcl-2* are no longer tumorigenic (Table 1). Mice that did not develop tumors were observed for 6–8 weeks.

DISCUSSION

***Bcl-2* Is an Important Component in *BCR–ABL*-Mediated Transformation.** The biological activities of the *BCR–ABL* oncogenes were unraveled by analyzing the effects of BCR–ABL expression on pro-B murine Ba/F3 cell growth. Ba/F3 cells ceased to proliferate and progressively died after IL-3 deprivation by a process of nuclear fragmentation characteristic of apoptosis. We have thus shown that, in the same experimental conditions, *BCR–ABL* expression protects Ba/F3 cells from activating their intrinsic suicide program, a situation that recalls the effect of other transforming proteins on survival (30, 31, 33). This biological activity of the BCR–ABL oncogenes in the Ba/F3 cell lines recapitulates a critical feature of the Ph^{1+} leukemia: accumulation of leukemic blasts with low intrinsic proliferation activity (27–29).

Our further findings define the molecular basis of *BCR–ABL* modulation of cell survival. Data presented here show that *Bcl-2*, which is not normally expressed in Ba/F3 cells, is activated by expression of *BCR–ABL* oncogenes and mediates the prevention of apoptosis in the Ba/F3 system. Moreover, modification of the growth and tumorigenicity of these *BCR–ABL*-expressing Ba/F3 cells occurs by altering the level of Bcl-2 expression. Our findings are consistent with a model in which Bcl-2 is downstream in the transformation signal pathway of *BCR–ABL* oncogenes. As further evidence of this, Bcl-2 expression is significantly reduced in these cells when expression of *BCR–ABL* oncogenes is inhibited both by using antisense RNA and also by transfecting with a zinc finger peptide, which blocks *BCR–ABL* transcription (34) (data not shown).

However, an increase in *Bcl-2* expression cannot be the only event involved in transformation by *BCR–ABL* oncogenes. The reasons are as follows. First, although *BCL2* plays an important role in tumorigenesis of t(14;18)-bearing lymphomas (e.g., in follicular lymphoma), deregulation of *BCL2* may not be oncogenic by itself (35). Second, constitutive *BCL2* expression does not result in growth factor-independent lines and therefore replaces not the growth signals induced via growth factor receptors but only the survival signals (36). Thus, other components in addition to Bcl-2 are required to reconstitute the *BCR–ABL* oncogene transformation signal. In this connection, the ability of *BCR–ABL* oncogenes to transform cells appears to be dependent on the function of *MYC*, although *MYC* overexpression does not substitute for the *BCR–ABL* oncogene transformation phenotype (22). Moreover, recent

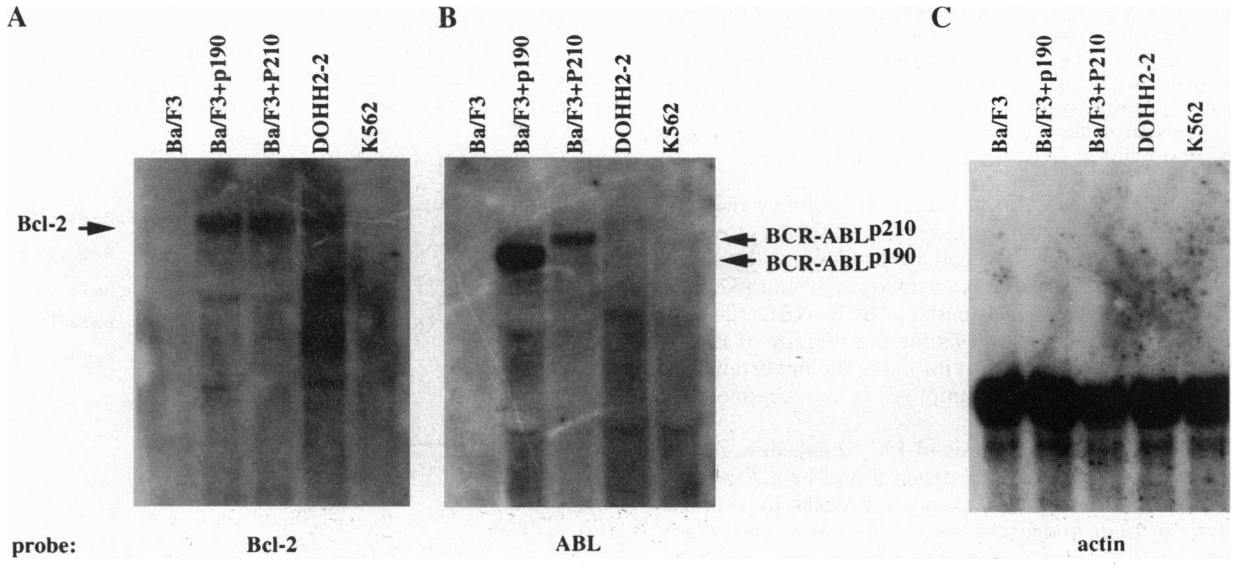

FIG. 2. *Bcl-2* expression in *BCR–ABL*-expressing cells. Normal-sized *Bcl-2* transcript of 7.5 kb is present in Ba/F3+p190 (lane 2), Ba/F3+p210 (lane 3), DoHH-2 (lane 4), and K562 (lane 5) cell lines. Cellular RNA was hybridized to a human *BCL2* cDNA probe (*A*), to an *ABL* probe (*B*), and to a mouse β-actin cDNA (*C*). Autoradiography was for 24 h at −70°C.

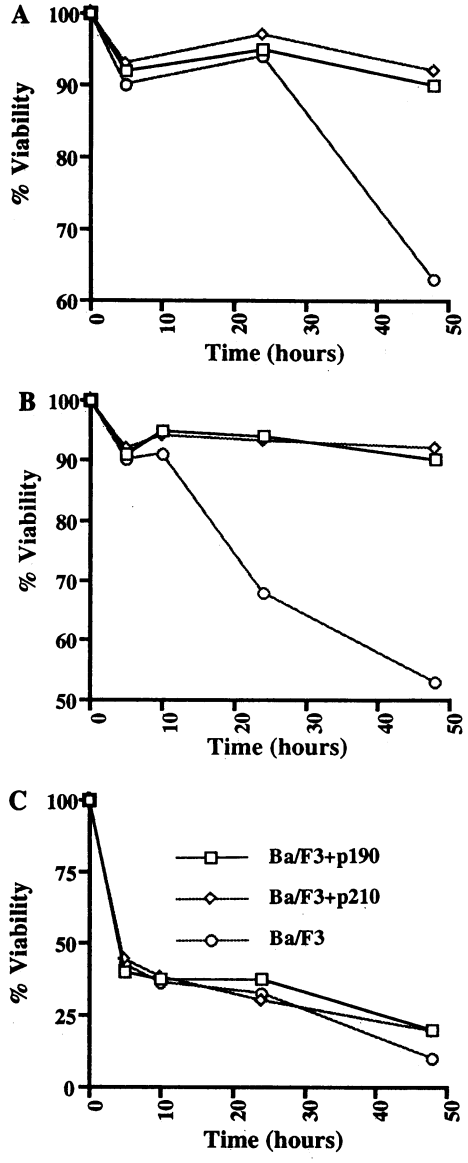

FIG. 3. Viability of transfected cells during hydrogen peroxidase-induced apoptotic cell death. Data are expressed as means of triplicate cultures. Percentage viability was determined by trypan blue exclusion. Quantitation of cells with apoptotic morphology yielded similar results. (*A*) Hydrogen peroxidase at 0.25 mM. Hydrogen peroxidase diluted 1:100 in phosphate-buffered saline was added to cell medium (5×10^5 cells per ml) at the beginning of the experiment. (*B*) Hydrogen peroxidase at 0.5 mM. (*C*) Hydrogen peroxidase at 1 mM.

studies with temperature-sensitive mutants to define the biological effects of *ABL* oncogenes have shown that the effect on apoptosis is an early event in cell transformation by these oncogenes, allowing time for a later oncogene event (37, 38).

The discovery that Bcl-2 is essential in BCR–ABL transformation may also have implications for the therapy of BCR–ABL-related human leukemias, reinforcing the importance of the therapeutic modulation of apoptosis in the treatment of human cancer.

***BCR–ABL* and the Pathogenesis of Ph^{1+} Leukemia.** Acute leukemia derives from the clonal expansion of hematopoietic precursors that have lost their capacity to proceed to terminal differentiation. Leukemogenesis would therefore require accumulation of the minimum number of genetic events that result in accelerated cell growth and differentiation block. A large number of genetic alterations, including specific chromosome translocations, have been identified and causally linked

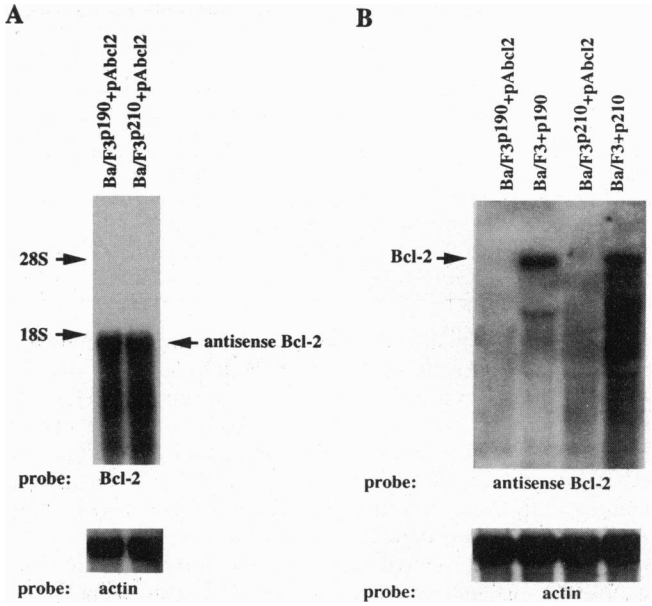

FIG. 4. Suppression of mediated *Bcl-2* RNA transcripts in *BCR–ABL*-expressing Ba/F3 cells transfected with pAbcl2. (*A*) Total RNA was isolated from Ba/F3+p190 (lane 1) and Ba/F3+p210 (lane 2) cells transfected with a vector expressing antisense mouse *Bcl-2* (pAbcl2). Cellular RNA was hybridized to a human *BCL2* cDNA probe. Autoradiography was for 9 h at −70°C. (*B*) Northern filter hybridization analysis of transfected (lanes 1 and 3) and untransfected (lanes 2 and 4) *BCR–ABL*-expressing Ba/F3 cells. An antisense mouse *Bcl-2* oligonucleotide was used as a probe. The antisense *Bcl-2* oligonucleotide used as a probe comprises the first 34 bases of the coding sequence of mouse *Bcl-2* cDNA. Autoradiography was for 24 h at −70°C.

to leukemogenesis, but the molecular basis of the composite leukemia phenotype remains largely unknown (39).

The property of *BCR–ABL* oncogenes of enhancing cell survival of Ba/F3 cells provides a cellular mechanism to explain their oncogenic action during Ph^{1+} leukemogenesis. The demonstration that *Bcl-2* is essential for transformation by *BCR–ABL* oncogenes, together with the fact mentioned above that these oncogenes also require *MYC* function (22), forces us to consider how the actions of these two genes might be connected. BCL2 has been found to cooperate with *MYC* in tumor induction or progression (31) and to antagonize MYC-induced apoptosis (40, 41). Therefore, the removal or blockage of BCR–ABL function might expose the cells to the apoptosis-inducing effects of MYC. Thus, one would expect that if the

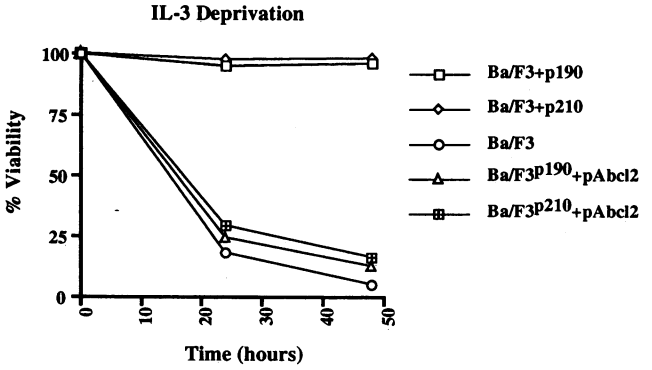

FIG 5. Viability of *BCR-ABL*-expressing cells transfected with pAbcl2 after IL-3 deprivation. Duplicate cultures of cells (5×10^5 cells per ml) were cultured in medium lacking IL-3, and living cells were counted at daily intervals. All values are normalized to the relative viability of the initial number of cells.

expression of BCR–ABL^{p190} is inhibited, apoptosis would reappear in these cells, and this has been observed (34). In contrast, when *BCR–ABL* is functional, this apoptotic pathway is blocked through Bcl-2 and the cells respond only to the proliferative function of MYC. Since MYC function is required for BCR–ABL-induced transformation (22), it is not unreasonable to suggest that BCR–ABL blocks MYC-induced apoptosis through Bcl-2. Indeed, recent studies have shown that cells harboring constitutively expressed forms of *MYC* are actually selected against, since inappropriate *MYC* expression activates the apoptotic program in the affected cell (42, 43). However, if the cell also contained an activating mutation in a gene that blocks apoptosis, the full transforming potential of a deregulated *MYC* gene would become evident. To date, the only other example of this type of cooperation with MYC is BCL2 itself (40, 41).

Oncoproteins that inhibit cell death (BCR–ABL, BCL2) cooperate with those that induce proliferation but cannot overcome death (MYC), which implies that cancer results from the combination of induction of proliferation and escape from death.

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